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14. ABSTRACT

We developed *in vitro* injury model of stroma cultured from bone marrow samples from mice and human donors. We confirmed that oxidative and hypoxic damage and estrogen deprivation can induce IL-6 and IL-8 export by human female, premenopausal stroma. We adapted a breast cancer cell co-culture model with injured stroma to determine the effect on stromal capacity to support dormancy. We adapted an osteoclast activation model to determine the capacity of injured stroma to activate osteoclasts. We also adapted a preosteoblast co-culture model to determine the role of osteoblasts in the micrometastatic cell niche responsible for recurrence of the dormant breast cancer. These experiments will serve to identify the adhesion and signaling necessary to maintain survival and resistance of dormant cancer cells.

15. SUBJECT TERMS

Bone marrow stroma, senescence, micrometastases, breast cancer, interleukin 6, osteoclast

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INTRODUCTION:

This year, we continued to investigate potential mechanisms for the reawakening of microscopic breast cancer micrometastastases in the bone marrow of patients at significant periods of time after initial treatment of clinically localized disease. We are testing the hypothesis that deprivation of estrogen that occurs after menopause may be a contributing factor to the generation of senile senescence in the bone marrow stroma of women. In this model, inflammatory cytokines generated by secretory senescent stromal cells induce dormant breast cancer cells to re-enter the cell cycle and grow into recurrent tumors in the bone. In the first year, we defined the secretory senescence phenotype initially in murine stroma and confirmed it in a human stromal sample using hypoxic and oxidative stress, and subsequently using estrogen deprivation. We developed *in vitro* stromal injury model in bone marrow stroma with hypoxia, oxidative stress and estrogen deprivation as defined by IL-6 and IL-8 export and activation of the TGFβ pathway. This year, we continued to collect male and female normal donor bone marrows and determined the response to injury and estrogen deprivation by cytokine production. We began developing co-cultivations methods for determining the effects of stromal injury on dormancy and proliferation of estrogen sensitive breast cancer cells. We also adapted methodology to determine the effect of injured stroma on osteoclast stimulation. Finally, we began to test a hypothesis that the niche harboring dormant breast cancer cells in the bone marrow involves osteoclast precursors that were shown to serve that role in hematopoietic stem cell survival.

BODY:

We continued some of our initial studies with murine stroma to characterize the system. We had previously demonstrated that the effects of 10, 100 and 1,000 μM hydrogen peroxide (H_2O_2), an agent that generates oxidative stress, of carbonyl-cyanide m-chlorophenylhydrazone (CCCP) an agent that blocks the mitochondrial electron transport chain, to create hypoxic stress, H_2O_2, and ICI182780, and estrogen receptor alpha (ERα) inhibitor at variable concentrations of 10^{-8}, 10^{-7} and 10^{-6} M induce inflammatory changes in murine and human stroma as measured by IL-6 and IL-8 export to the media and SMAD-2 and SMAD-3 phosphorylation. Now we show in Figure 1 that estrogen deprivation in murine stroma also induces inflammation as demonstrated by TNFα phosphorylation.

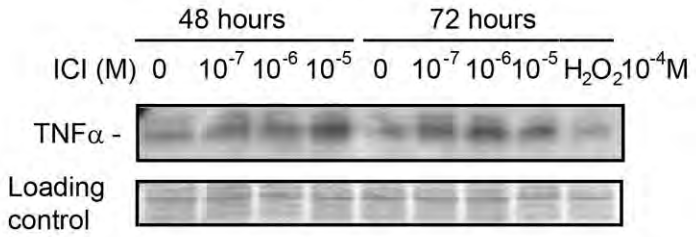


Figure 1. Western blot for phospho-TNFα of mouse stromal cells treated with estrogen deprivation. Nearly confluent mouse stromal monolayers maintained in 10 cm plastic dishes were treated with ICI 182780 of 10^{-7}, 10^{-6} and 10^{-5} M for 48 hr and 72 hr were lysed in modified RIPA buffer and analyzed by SDS-PAGE with antibody to mouse phospho-phospho-TNFα (Cell Signaling). Uniform bands from Coomassie Blue-stained membranes were used to verify equal loading. The data demonstrate increased phospho-TNFα staining with two and three days of estrogen deprivation.

Our initial experiments with human stroma demonstrated statistically significant differences in IL-6 export between control and CCCP-treated cells occurred during most of the time points from 1 to 28 days and estrogen-deprived stroma after a week of incubation that was sustained for the

28 days assayed in one premenopausal donor. We obtained bone marrow from a total of 8 normal volunteer donors, 6 premenopausal females and 2 two male controls, under a UMDNJ and US Army IRB-approved protocol. No postmenopausal female subjects volunteered for the study.

The methods are well established in our lab and are as follows: Aspirates were centrifuged at 1,500 rpm for 15 minutes and buffy coats were collected and cultured in 25cm^2 flask in complete McCoy's medium. Human stroma grow at a far slower rate than murine stroma, with human stroma obtained from 20 ml of bone marrow aspirate requiring 8 weeks to be sufficiently confluent to permit passage to 24 well plates. After 8 weeks the cells were trypsinized and distributed onto all of the wells of a 24 well plate and permitted to grow to near confluence for about 2 weeks.

Confluent monolayers of human stromal cells on 24 well plates were treated with H_2O_2, CCCP and ICI182780 in quadruplicate. Rows of 4 wells were either untreated or treated with 100 μM H_2O_2, 100μM CCCP for 1 hr at 37^o, washed with PBS and then replenishes with McCoy's complete growth medium. To the fourth row, 10^{-6} M ICI182780 in phenol red-free medium was added, in which cells were maintained throughout the duration of the experiment. Supernatants from each well were collected at specified time points and stored at -80^o. ELISAs were performed on thawed conditioned media for IL-6 and IL-8 using a Human IL-6 Elisa Kit from BD & Human CXCL8/IL-8 kit from R&D Systems respectively. Control wells are the wells in which McCoy's media was replaced by DMEM/10% FCS.

Here we present data from three additional female premenopausal volunteers and one male volunteer. The data from Figure 2 A-D are summarized in Table 1.

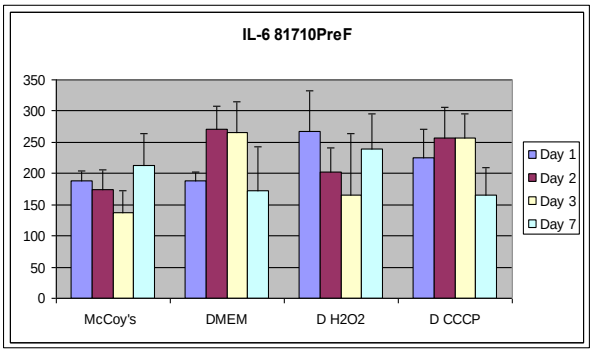
TABLE 1

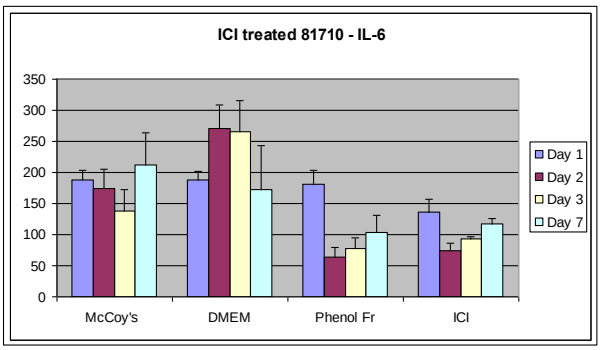
	Subject	H_2O_2	CCCP	ICI182780
A.	81710PreF			
	IL-6	+ 1d	+ 1d	+ 21d, 28d
	IL-8	-	-	+ 3d, 7d
B.	121319PreF			
	IL-6	+ 1d, 2d, 3d	+ 1d, 2d, 3d	+ 7d, 28d weakly
	IL-8	-	-	+ 1, 2, 3, 7, 14, 21, 28d
C.	121710PreF			
	IL-6	+ 3d	+ 3d, 7d	+ 3, 7 and +weakly 28d
	IL-8	+ 2, 3, 3d	+ 3d weakly	+ 1, 2d
D.	111710M			
	IL-6	-	-	-
	IL-8	-	-	-

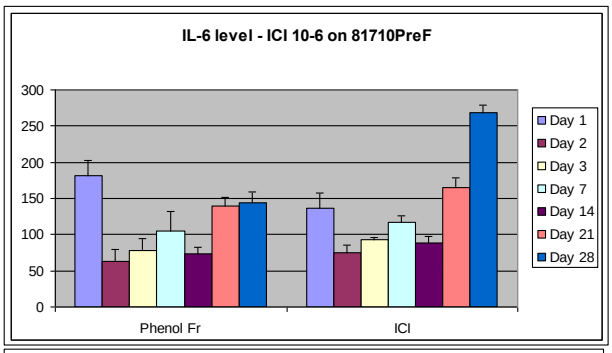
These data appear to demonstrate a relatively consistent pattern of response, although the timing of the response varies somewhat. IL-6 in the conditioned media consistently increases with H_2O_2 and CCCP one hour treatment from 1-3 days in the three female subjects and increases with estrogen deprivation most frequently between 7-28 days. IL-8 secretion responds infrequently to oxidative and hyoxic stress but is reliably exported in response to estrogen deprivation in females. The one male assessed did not produce IL-6 nor IL-8 to any of the stimuli. It remains to be confirmed whether male and female stroma respond differently to injury under the conditions tested.

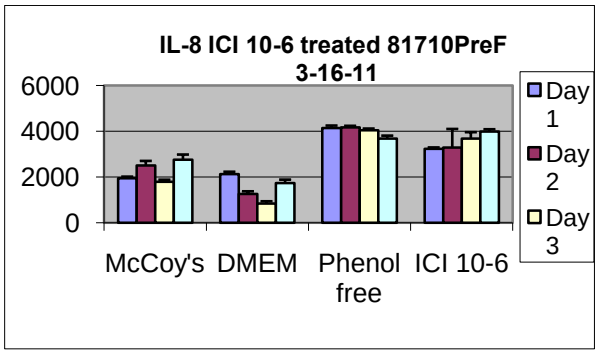
Figure 2.

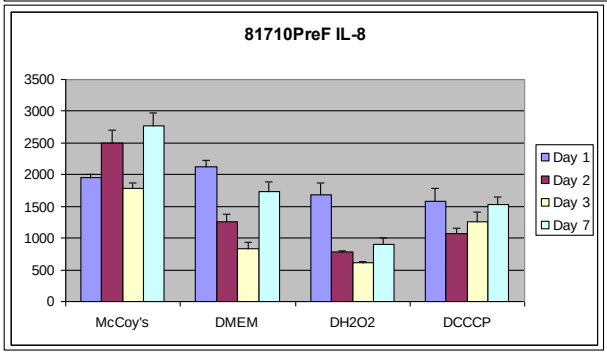
A.


IL-6 81710PreF
350
300
250
200
150
100
50
0
McCoy's
DMEM
D H2O2
D CCCP
Day 1
Day 2
Day 3
Day 7

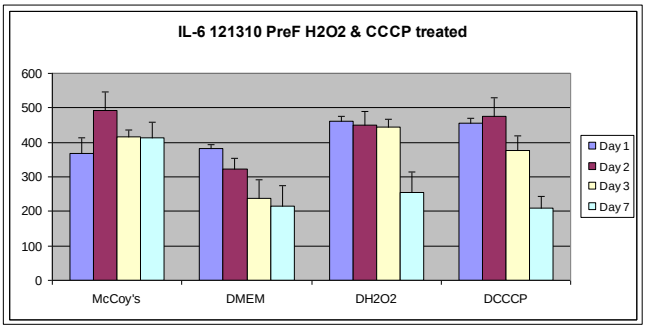

ICI treated 81710 - IL-6
350
300
250
200
150
100
50
0
McCoy's
DMEM
Phenol Fr
ICI
Day 1
Day 2
Day 3
Day 7

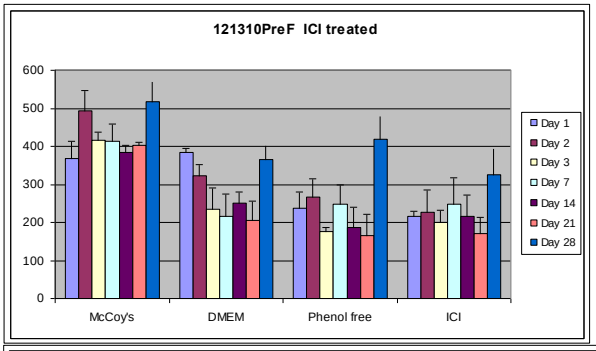

IL-6 level - ICI 10-6 on 81710PreF
300
250
200
150
100
50
0
Phenol Fr
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

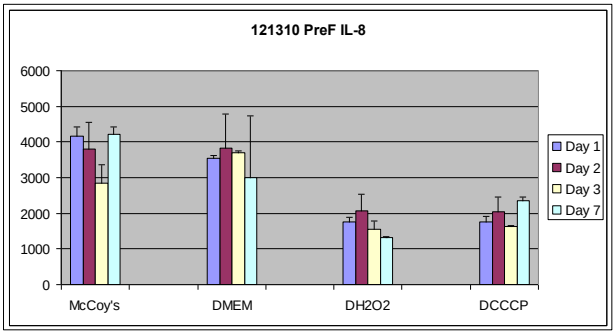

IL-8 ICI 10-6 treated 81710PreF
3-16-11
6000
4000
2000
0
McCoy's
DMEM
Phenol free
ICI 10-6
Day 1
Day 2
Day 3

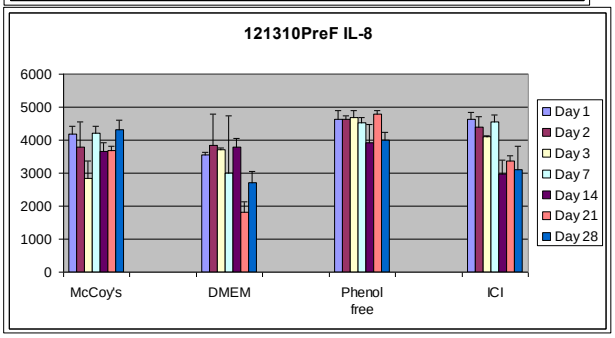

81710PreF IL-8
3500
3000
2500
2000
1500
1000
500
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7

B.

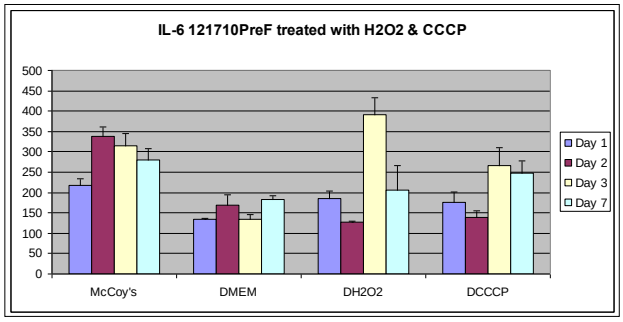

IL-6 121310 PreF H2O2 & CCCP treated
600
500
400
300
200
100
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7

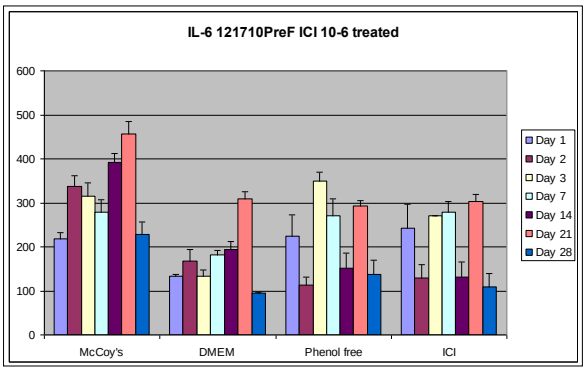

121310PreF ICI treated
600
500
400
300
200
100
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

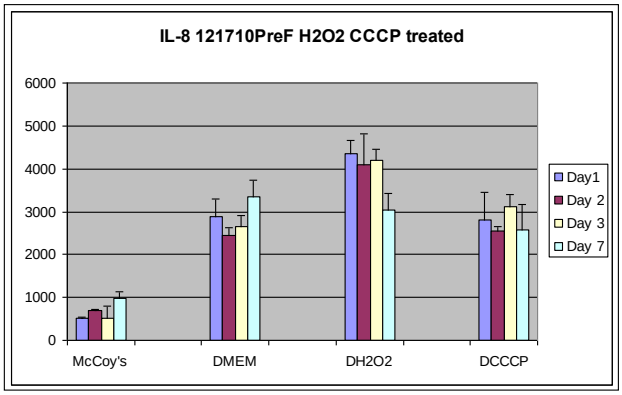

121310 PreF IL-8
6000
5000
4000
3000
2000
1000
0
McCoy's
DMEM
DH2O2
DCCCP
Day 1
Day 2
Day 3
Day 7

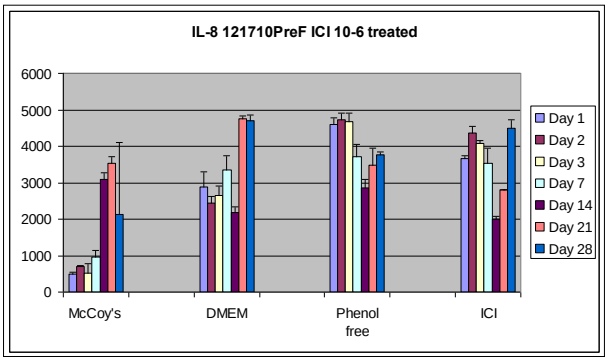

121310PreF IL-8
6000
5000
4000
3000
2000
1000
0
McCoy's
DMEM
Phenol free
ICI
Day 1
Day 2
Day 3
Day 7
Day 14
Day 21
Day 28

C.

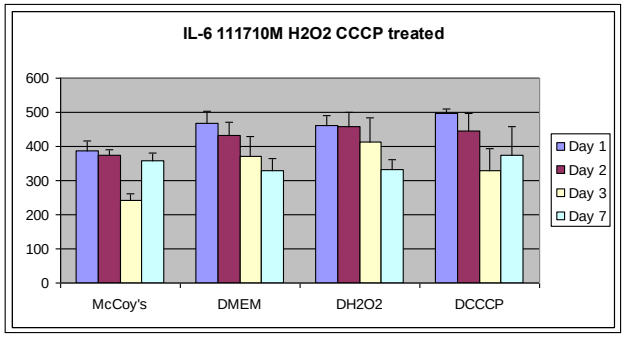








D.



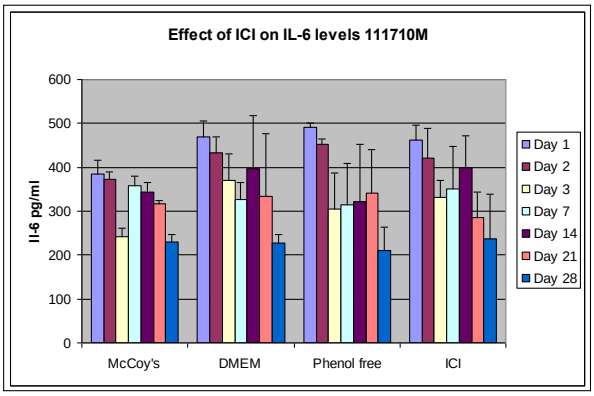


Figure 2. Export of IL-6 and IL-8 in human bone marrow stroma in response to stimulation by H_2O_2, CCCP and estrogen deprivation. Measurements of IL-6 and IL-8 in conditioned media from stroma incubated in 24 well tissue culture plates. McCoy's media used to culture stroma to confluence was replaced with DMEM/10% FCS supplemented with H_2O_2, CCCP 100 μM for one hour or in continuous culture with with Phenol Red-free media with or without supplemented 10^{-6} M ICI182780. Conditioned media were collected at the times indicated and stored at -80°C, thawed collectively assayed at the same time for IL-6 and IL-8 using a Human IL-6 Elisa Kit from BD & Human CXCL8/IL-8 kit from R&D Systems respectively. Control wells are ones in which McCoy's media was replaced by DMEM/10% FCS.

We began efforts to determine if the inflammatory phenotype of injured stroma promotes the growth of dormant breast cancer clones. We adapted a co-culture model with breast cancer cells to determine if the ratio of dormant to growing clones changes with stromal injury. To visualize the breast cancer cells on stromal monolayers, we labeled MCF-7 cells with Cell Tracker fluorescent probes (Invitrogen) using a variety of colors. Cells were incubated on confluent stromal monolayers that were injured 2 days prior with H_2O_2 or CCCP 100 μM for one hour or in continuous culture with Phenol Red-free media for 7 days with supplemented 10^{-6} M ICI182780 and had media changed to DMEM/10% FCS prior to co-incubation. After 6 days in co-culture, cells on stroma were photographed by phase contrast and fluorescence microscopy. Figure 3 demonstrates that single

cells predominate on unmanipulated stroma wile clumps of fluorescent cell cultures appear on injured stroma. These experiments are preliminary and we do not have quantitation as of yet. We will be counting dormant and growing breast cancer cell clones on the stroma and determining if stromal injury affects their ratio.

Figure 3. MCF-7 cells on Human Stroma

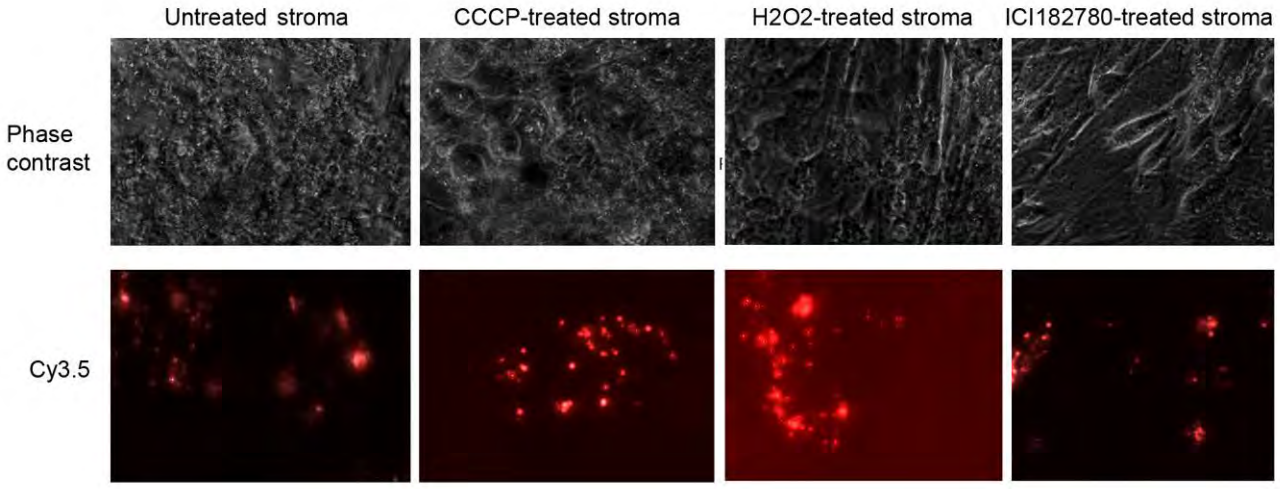


Figure 3. Human bone marrow stromal cultures from a pre-menopausal female were grown to near confluence on 125 mm flasks and transferred to 24 well tissue culture plates by trypsinization and grown to confluence. Confluent monolayers were incubated with MCF-7 cells labeled with Cell Tracker fluorescent probe (Invitrogen) for six days. Cells were photographed under phase contrast and fluorescence microscopy. The data show more single breast cells on the untreated stroma and clumps of cells on injured or estrogen-deprived stroma. These are initial observations and quantitative assessment is not yet available. It does demonstrate that this method can be used to assess the effect of stromal injury on in vitro dormancy support by stroma.

We proceed to investigate the effects of stromal injury on mechanisms of dormant breast cancer reawakening by trying to determine the effects of injured stroma conditioned media on osteoclast activation. To determine this effect, we adapted an osteoclast assay to quantitatively measure response of osteoclasts to stimuli. We used BD BioCoat™ Osteologic™ Discs that incorporate a resorbable artificial bone analog in the form of sub-micron calcium phosphate films on transparent quartz substrates. This system can be used as a method for direct assessment of osteoclast activity *in vitro*. We used primary osteoclasts from athymic mouse bone marrows without further purification. Bone marrow was flushed from mouse femurs with 1 ml Osteoclast medium consisting of αMEM containing 15% FBS/Penn/Strep. The cell suspension was diluted to different dilutions with αMEM containing 15% FBS/Penn/Strep, 0.28 mM L-Ascorbic Acid 2-Phosphate and 10 mM β-Glycerophosphate and 1 ml was added on to each Disc placed in a 24 well dish and incubated at 37^{o}, 5% CO_2, 100% humidity. The medium was changed after 24 hours and then 3 times a week. The osteoclast cultures were maintained for 8-10 days, after which the medium was removed and the discs washed with Milli-Q water. 1ml of bleach solution (~6% NaOCl, ~5.2% NaCl) was added to each well. The solution was pipetted up and down to dislodge the cells. The bleach was aspirated after 5 minutes at room temperature and the discs were washed 3 times with ~2ml of distilled water. The discs were allowed to air dry and examined under the microscope. Figure 4 demonstrates the osteoclast discs and the osteoclast-induced lacunae after an 8-10 day incubation. We demarkated maximal longitudinal diameters in preparation of quantitative assessment of conditioned-media effect on osteoclast activity. We plan to incubate bone marrow with conditioned media on the discs for 24 hours prior to the first change of osteoclast media.

Figure 4.

MC3T3E1 MC3T3E1 - MCF-7 co-culture Mouse Osteoclasts on Disc

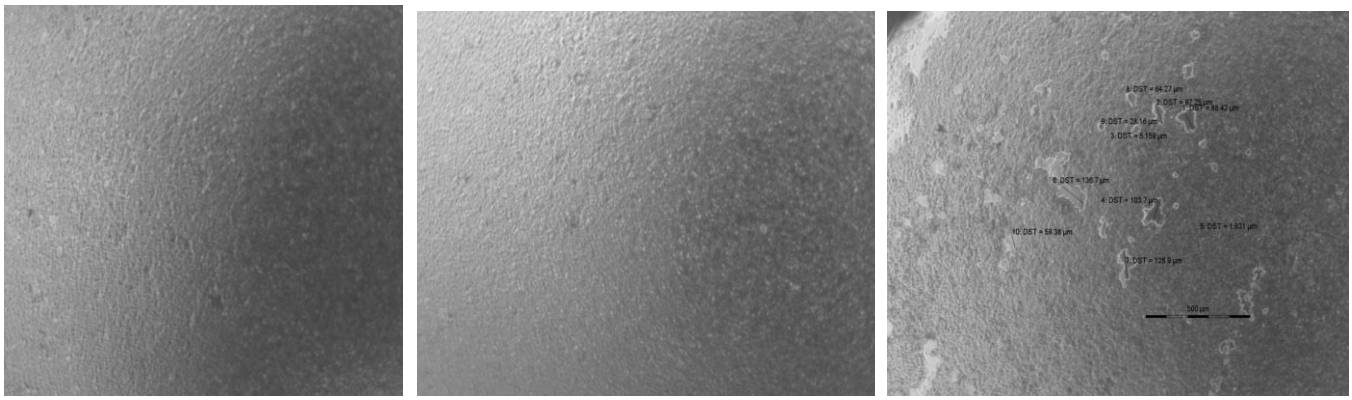


Figure 4. BD BioCoat™ Osteologic™ Discs incubated with Rat Osteoblast cell line MC3T3E1, MC3T3E1 and MCF-7 cells and mouse bone marrow containing osteoclasts. Cells were cultured on discs for 10 days in αMEM containing 15% FBS/Penn/Strep, 0.28 mM L-Ascorbic Acid 2-Phosphate and 10 mM β-Glycerophosphate. Mouse osteoclasts produced numerous, measurable lacunae at a high efficiency while MC3T3 E1 (Preosteoblasts) and MCF-7 did not produce significant numbers of sizes of lacunae. The osteoclast-induced lacunae have longitudinal measurement bars demonstrating that we will be able to quantitate size as well as number differences when we stimulate osteoclasts with stromal conditioned medium.

Another aspect of the project that is highly relevant to our question is the nature of the microenvironement in the bone marrow that supports dormancy of breast cancer cells with the capacity to regrow into recurrent tumors. The nature of this niche has been demonstrated in hematopoietic stem cells to be directly dependent on preosteoblasts lining the bone marrow cavity in the bone marrow stroma. These osteoblasts are necessary for hematopoietic stem cell survival and a genetically induced increase in their number increases the number of hematopoietic stem cells. We hypothesized that osteoblasts also provide a niche for breast cancer repopulating micrometastases in the bone marrow. We proceeded to investigate this possibility in vitro using the rat calvarium preosteoblast line MC3T3E1. We formed monolayers of MC3T3E1 cells as well as monolayers of mouse stromal cells to determine the relative efficiency of MCF-7 human breast cancer cell dormant colony formation. Figure 5 demonstrated the appearance of the osteoblast and stromal monolayers and the presence of growing and dormant MCF-7 breast cancer clones on them after 6 days in coculture. Breast cancer cells were labeled with Invitrogen Cell Tracker fluorescent probes for visualization.

Figure 5. MCF-7 clone formation on various substrata

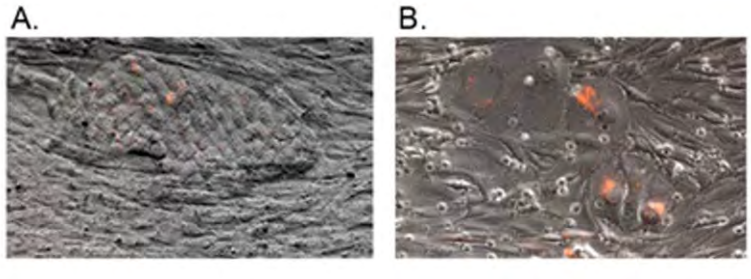


Figure 5. MCF-7 breast cancer cells labeled with Cell Tracker (Invitrogen) were incubated for 6 days on confluent monolayers of rat calvarium preosteoblast line MC3T3E1 (A) and mouse bone marrow stroma (B). **A.** Growing (left) and dormant (right) clones on a monolayer of osteoblasts. **B.** Dormant clones on a monolayer of stroma. All MCF-7 cells were labeled with Cell Tracker cell-labeling solution prior to co-incubation on the various substrata.

We were able to quantitate the number of growing (>30 cells estimated) and dormant (<12 cell estimated) clones after 6 days in culture in a preliminary experiments. While stroma produce their own FGF-2, as we have previously demonstrated, we added exogenous FGF-2 10 ng/ml to half the cultures because osteoblasts were negative for FGF-2 by Western blot. Triplicate wells were counted for numbers of growing and dormant clones. Counting was conducted by switching between phase contrast and fluorescent imaging of the same fields to confirm the morphology of the colony containing fluorescent cells. Growing clones had much lower intensity fluorescence due to dilution of the labels with each cell division, hence accounting for only a fraction of cells with detectable fluorerscence. Figure 6 demonstrates the numbers of dormant to growing clones on osteoblasts, stroma and in 10:1 mixture of stroma to osteoblasts.

Figure 6. The effect of bone marrow stroma and osteoblasts on dormant clone formation

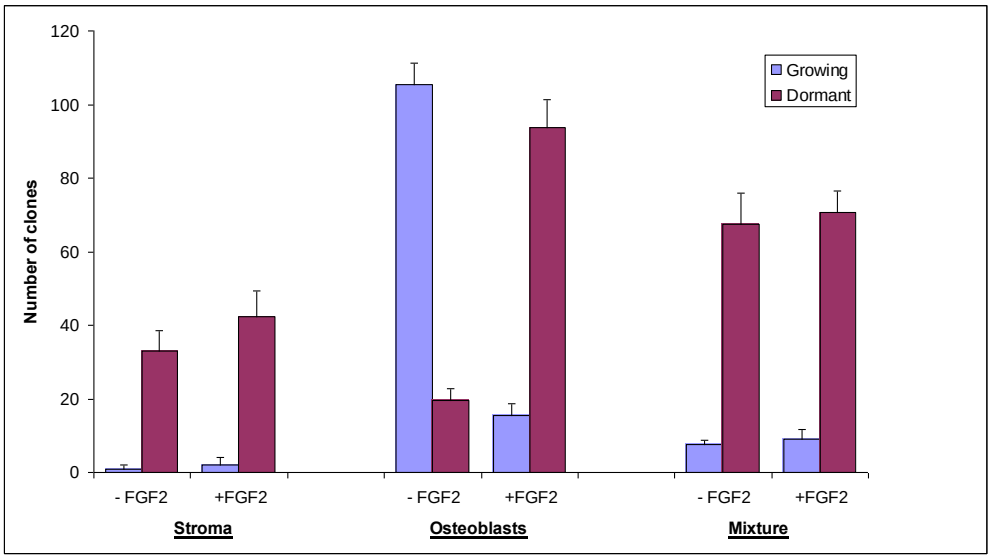


Figure 6. Numbers of growing (>30 cells estimated) and dormant (<12 cell estimated) clones after 6 days in culture of MCF-7 cells incubated on confluent monolayers of athymic mouse bone marrow stroma, on rat calvarium preosteoblast line MC3T3E1 and on a 10:1 mixture of stroma to osteoblast cells. MCF-7 cells were labeled with Cell Tracker (Invitrogen) and colonies were visualized by imaging by both phase contrast and fluorescence microscopy. As previously documented by our group, there were very few growing clones on stroma, with a significant number of dormant clones. The number of dormant clones did not increase with FGF-2 treatment, as expected. Osteoblasts do not produce FGF-2 and consequently, very few dormant clones were observed in its absence. They did support highly efficient growing clone formation. Addition of FGF-2 to the osteclast cocultures induced a robust dormant clone formation at double the efficiency of that on stroma. Addition of 1/10 osteoblasts to a mixed stromal monolayer also increased the efficiency of dormant clone formation by nearly a factor of 2 while stimulating the formation of growing clones as well.

The ratios of dormant to growing MCF-7 clones are depicted in tables 2-4. Ratios of dormant to growing MCF-7 clones were unchanged ($p>0.05$) regardless of treatment with exogenous FGF-2 on stroma and a mixture of 10:1 stroma:osteoblast monolayers. However, on a monolayer of osteoblasts alone, the ratio of dormant to growing clones was significantly reduced when left untreated with exogenous FGF-2 ($p<0.05$). Error bars indicate ± SD.

Table 2. Number of growing MCF-7 clones on various substrata without exogenous FGF-2

	Number of growing clones	Change compared to plastic and fibronectin	P-value compared to plastic	P-value compared to fibronectin
Plastic	66		-	0.018
Fibronectin	39		0.018	-
Osteoblasts	105	increased	0.001	<0.001
Stroma	1	decreased	<0.001	0.004
Mixture	8	decreased	<0.001	0.008

Without exogenous FGF-2, osteoblasts promote growth of MCF-7 cells. Stromal and mixed cultures inhibit growth.

Table 3. Number of dormant MCF-7 clones on osteoblasts and stroma

	Number of dormant clones without FGF-2	Number of dormant clones with FGF-2	P-value, without versus with FGF-2
Osteoblasts	20	94	<0.001
Stroma	33	42	0.15

Osteoblasts require exogenous FGF-2 to induce dormancy of MCF-7 cells. Bone marrow stroma does not require it, likely due to endogenous FGF-2 being produced and released by stromal cells.

Table 4. Number of dormant MCF-7 clones on osteoblasts, stroma, and mixture

	Number of dormant clones without FGF-2	Number of dormant clones with FGF-2
Stroma	33 *	42 * *
Osteoblasts	20 *	94 *
Mixture	67	71

* Indicates statistical significance ($p<0.05$)

Without exogenous FGF-2, osteoblasts support fewer dormant clones than stroma. However, when exogenous FGF-2 is provided, the presence of osteoblasts, either alone or in the 10:1 stroma to osteoblast mixture, significantly increases the number of dormant clones.

These data suggest that osteoblasts alone do not appear to support dormancy of breast cancer cells when FGF-2 is absent. However, when provided with FGF2 either exogenously with recombinant FGF2 or through mixing with bone marrow stromal cells, osteoblasts appear to promote increased dormant clone formation.

Since osteoblasts in the bone marrow niche are in the presence of FGF-2 produced by contiguous stromal cells, the scenario observed in the mixing experiments is likely more representative than that of osteoblast co-cultures alone. These mixing experiments suggests that the presence of osteoblasts in a stromal microenvironment increases the rate of dormancy compared with that induced by stroma alone. Similarly, osteoblasts increase dormant colony formation in pure cocultures with exogenous FGF2 added. The mechanism of osteoblast induced greater number of dormant clone formation as well as that of increased growing clone formation will be important to determine. We have demonstrated that increased survival of dormant clones on bone marrow stroma is in part due to activation of the PI3 kinase pathway. It is likely that activation of a parallel pathway is induced by adhesion molecule signaling activated by osteoblast ligation, but these data are yet to be determined.

It will also be important to determine the capacity of dormant clones in these co-cultures to repopulate a tumor and whether the enhanced dormant clone formation on osteoblasts correlates with greater repopulating capacity of those cells.

KEY RESEARCH ACCOMPLISHMENTS:

- Developed *in vitro* stromal injury model in mouse and human bone marrow stroma
- Confirmed that H_2O_2-induced oxidative damage and CCCP-induced hypoxic damage can induce IL-6 and variably IL-8 export by human stroma
- Demonstrated that estrogen deprivation of human bone marrow stroma induces export of IL-6 and IL-8 in female, premenopausal stroma, but possibly not in male stroma
- Adapted a co-culture model of breast cancer cells with injured stroma to determine the effect on sromal capacity to support dormancy
- Adapted an osteoclast activation model to determine the effect of injured stroma to activate osteoclasts
- Adapted a preosteoblast co-culture model to determine the role of osteoblasts in the micrometastatic cell niche responsible for recurrence of the dormant breast cancer

REPORTABLE OUTCOMES:

The data is being presented at the Department of Defense Era of Hope Breast Cancer Meeting in Orlando Florida August 2-5, 2011.

CONCLUSIONS:

We hypothesized that estrogen deprivation may be one mechanism that can induce secretory senescence in the bone marrow stroma, and effect that may be universal in menopause. Here, we demonstrated that injuring human female premenopausal stroma with hypoxia, oxidative stress and estrogen deprivation induces the secretion of inflammatory cytokines to its microenvironment. This is potentially of great significance, as the secreted inflammatory cytokines may induce breast cancer micrometastases that have been dormant in the stromal microenvironment for extended periods to begin proliferating again and result in incurable recurrent disease.

We adapted existing techniques to test the effects of this inflammatory phenotype on the capacity of stroma to support dormant clone formation. By using Cell Tracker and combining phase

contrast and fluorescence imaging, we can identify dormant and growing breast cancer clones on bone marrow stroma. Experiments will be conducted to determine the effects of stromal injury on reversal of dormancy. Blocking experiments will determine the signaling pathways and cytokines necessary to induce these effects.

We adapted an assay to determine the effect of injured stroma to activate osteoclasts. These studies will be an important adjunct to the reactivation of dormant clone formation assays because they address another aspect of recurrence, that of the activation of the vicious cycle of bone erosion and a potential mechanism of reversing it.

We began to address the specific niche in the microenvironment responsible for support of the micrometastatic dormant clone. We used preosteoblast cell line to co-culture breast cancer cells alone and in mixing experiments with sromal cells. Our preliminary experiments demonstrated that osteoblasts promote the growth of both growing as well as dormant clones. Confirmation of these studies and determining mechanisms for these effect will be key in understanding the survival effects of the niche on dormant micrometastases. The characteristic cancer repopulating capacity of breast cancer cells forming dormant clones on osteoblasts will be key in addressing this specific, chemo and radioresistant population.

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APPENDICES:

None

SUPPORTING DATA:

All data appear in the body.